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PCT International Application Processing Div.
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Re: International Application No. PCT/US03/17941
Title: METHODS OF DIAGNOSING & TREATING DIABETES AND INSULIN RESISTANCE
Applicant: METABOLEX, INC. *et al.*
International Filing Date: 4 June 2003
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Dear Officer:

Enclosed are the Chapter II Demand and five (5) substitute specification pages 23, 24, 43, 44 and 80 submitted as an Article 34 Amendment for the above-referenced patent application. The only changes were insertions of SEQ ID:NOs. and corrections of typographical errors that do not include matter which go beyond the disclosure in the international application as filed.

Pursuant to Rule 92*bis*, Applicants respectfully request the recordation of a change in the Applicant's, Metabolex, Inc., telephone and facsimile numbers. Please update:

Telephone number from 510.293.8828 to 510.293.8800
Facsimile number from 510.293.6853 to 510.293.9090

Applicants respectfully await the Notification of the Recording of a Change, Form PCT/IB/306.

Thank you for your attention to this matter.

Respectfully submitted,

TOWNSEND and TOWNSEND and CREW LLP

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JML/nan
Enclosures: Chapter II Demand w/ five (5) substitute specification pages 23, 24, 43, 44 and 80
Forty-nine (49) pages of Sequence Listing, Diskette and Statement
Transmittal Letter and Postcard

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the cell culture media) from the recombinant protein of interest. The preferred salt is ammonium sulfate. Ammonium sulfate precipitates proteins by effectively reducing the amount of water in the protein mixture. Proteins then precipitate on the basis of their solubility. The more hydrophobic a protein is, the more likely it is to precipitate at lower ammonium sulfate concentrations. A typical protocol is to add saturated ammonium sulfate to a protein solution so that the resultant ammonium sulfate concentration is between 20-30%. This will precipitate the most hydrophobic proteins. The precipitate is discarded (unless the protein of interest is hydrophobic) and ammonium sulfate is added to the supernatant to a concentration known to precipitate the protein of interest. The precipitate is then solubilized in buffer and the excess salt removed if necessary, through either dialysis or diafiltration. Other methods that rely on solubility of proteins, such as cold ethanol precipitation, are well known to those of skill in the art and can be used to fractionate complex protein mixtures.

2. Size Differential Filtration

[0081] Based on a calculated molecular weight, a protein of greater and lesser size can be isolated using ultrafiltration through membranes of different pore sizes (for example, Amicon or Millipore membranes). As a first step, the protein mixture is ultrafiltered through a membrane with a pore size that has a lower molecular weight cut-off than the molecular weight of the protein of interest. The retentate of the ultrafiltration is then ultrafiltered against a membrane with a molecular cut off greater than the molecular weight of the protein of interest. The recombinant protein will pass through the membrane into the filtrate. The filtrate can then be chromatographed as described below.

3. Column Chromatography

[0082] The proteins of interest can also be separated from other proteins on the basis of their size, net surface charge, hydrophobicity and affinity for ligands. In addition, antibodies raised against proteins can be conjugated to column matrices and the proteins immunopurified. All of these methods are well known in the art.

[0083] Immunoaffinity chromatography using antibodies raised to a variety of affinity tags such as hemagglutinin (HA), FLAG, Xpress, Myc, hexahistidine (SEQ ID NO:35) (His), glutathione S transferase (GST) and the like can be used to purify polypeptides. The His tag will also act as a chelating agent for certain metals (e.g., Ni) and thus the metals can also be

used to purify His-containing polypeptides. After purification, the tag is optionally removed by specific proteolytic cleavage.

[0084] It will be apparent to one of skill that chromatographic techniques can be performed at any scale and using equipment from many different manufacturers (*e.g.*, Pharmacia Biotech).

IV. DETECTION OF POLYNUCLEOTIDES OF THE INVENTION

[0085] Those of skill in the art will recognize that detection of expression of polynucleotides and polypeptides of the invention has many uses. For example, as discussed herein, detection of levels of polynucleotides and polypeptides of the invention in a patient is useful for diagnosing diabetes or a predisposition for at least some of the pathological effects of diabetes. Moreover, detection of gene expression is useful to identify modulators of expression of polynucleotides and polypeptides of the invention.

[0086] A variety of methods of specific DNA and RNA measurement that use nucleic acid hybridization techniques are known to those of skill in the art (*see*, Sambrook, *supra*). Some methods involve an electrophoretic separation (*e.g.*, Southern blot for detecting DNA, and Northern blot for detecting RNA), but measurement of DNA and RNA can also be carried out in the absence of electrophoretic separation (*e.g.*, by dot blot). Southern blot of genomic DNA (*e.g.*, from a human) can be used for screening for restriction fragment length polymorphism (RFLP) to detect the presence of a genetic disorder affecting a polypeptide of the invention.

[0087] The selection of a nucleic acid hybridization format is not critical. A variety of nucleic acid hybridization formats are known to those skilled in the art. For example, common formats include sandwich assays and competition or displacement assays. Hybridization techniques are generally described in Hames and Higgins *Nucleic Acid Hybridization, A Practical Approach*, IRL Press (1985); Gall and Pardue, *Proc. Natl. Acad. Sci. U.S.A.*, 63:378-383 (1969); and John *et al. Nature*, 223:582-587 (1969).

[0088] Detection of a hybridization complex may require the binding of a signal-generating complex to a duplex of target and probe polynucleotides or nucleic acids. Typically, such binding occurs through ligand and anti-ligand interactions as between a ligand-conjugated probe and an anti-ligand conjugated with a signal. The binding of the signal generation complex is also readily amenable to accelerations by exposure to ultrasonic energy.

support, and the tagged molecule of interest is attached to the solid support by interaction of the tag and the tag binder.

[0159] A number of tags and tag binders can be used, based upon known molecular interactions well described in the literature. For example, where a tag has a natural binder, for example, biotin, protein A, or protein G, it can be used in conjunction with appropriate tag binders (avidin, streptavidin, neutravidin, the Fc region of an immunoglobulin, poly-His, *etc.*) Antibodies to molecules with natural binders such as biotin are also widely available and appropriate tag binders (*see,* SIGMA Immunochemicals 1998 catalogue SIGMA, St. Louis MO).

[0160] Similarly, any haptenic or antigenic compound can be used in combination with an appropriate antibody to form a tag/tag binder pair. Thousands of specific antibodies are commercially available and many additional antibodies are described in the literature. For example, in one common configuration, the tag is a first antibody and the tag binder is a second antibody that recognizes the first antibody. In addition to antibody-antigen interactions, receptor-ligand interactions are also appropriate as tag and tag-binder pairs, such as agonists and antagonists of cell membrane receptors (*e.g.,* cell receptor-ligand interactions such as transferrin, c-kit, viral receptor ligands, cytokine receptors, chemokine receptors, interleukin receptors, immunoglobulin receptors and antibodies, the cadherin family, the integrin family, the selectin family, and the like; *see, e.g.,* Pigott & Power, *The Adhesion Molecule Facts Book I* (1993)). Similarly, toxins and venoms, viral epitopes, hormones (*e.g.*, opiates, steroids, *etc.*), intracellular receptors (*e.g.*, which mediate the effects of various small ligands, including steroids, thyroid hormone, retinoids and vitamin D; peptides), drugs, lectins, sugars, nucleic acids (both linear and cyclic polymer configurations), oligosaccharides, proteins, phospholipids and antibodies can all interact with various cell receptors.

[0161] Synthetic polymers, such as polyurethanes, polyesters, polycarbonates, polyureas, polyamides, polyethyleneimines, polyarylene sulfides, polysiloxanes, polyimides, and polyacetates can also form an appropriate tag or tag binder. Many other tag/tag binder pairs are also useful in assay systems described herein, as would be apparent to one of skill upon review of this disclosure.

[0162] Common linkers such as peptides, polyethers, and the like can also serve as tags, and include polypeptide sequences, such as poly-Gly sequences of between about 5 and 200

amino acids (SEQ ID NO:36). Such flexible linkers are known to those of skill in the art. For example, poly(ethylene glycol) linkers are available from Shearwater Polymers, Inc., Huntsville, Alabama. These linkers optionally have amide linkages, sulfhydryl linkages, or heterofunctional linkages.

[0163] Tag binders are fixed to solid substrates using any of a variety of methods currently available. Solid substrates are commonly derivatized or functionalized by exposing all or a portion of the substrate to a chemical reagent that fixes a chemical group to the surface that is reactive with a portion of the tag binder. For example, groups that are suitable for attachment to a longer chain portion would include amines, hydroxyl, thiol, and carboxyl groups. Aminoalkylsilanes and hydroxyalkylsilanes can be used to functionalize a variety of surfaces, such as glass surfaces. The construction of such solid phase biopolymer arrays is well described in the literature (*see, e.g.*, Merrifield, *J. Am. Chem. Soc.* 85:2149-2154 (1963) (describing solid phase synthesis of, *e.g.*, peptides); Geysen *et al., J. Immun. Meth.* 102:259-274 (1987) (describing synthesis of solid phase components on pins); Frank and Doring, *Tetrahedron* 44:60316040 (1988) (describing synthesis of various peptide sequences on cellulose disks); Fodor *et al., Science*, 251:767-777 (1991); Sheldon *et al., Clinical Chemistry* 39(4):718-719 (1993); and Kozal *et al., Nature Medicine* 2(7):753759 (1996) (all describing arrays of biopolymers fixed to solid substrates). Non-chemical approaches for fixing tag binders to substrates include other common methods, such as heat, cross-linking by UV radiation, and the like.

[0164] The invention provides *in vitro* assays for identifying, in a high throughput format, compounds that can modulate the expression or activity of a polypeptide of the invention. Control reactions that measure activity of a polypeptide of the invention in a cell in a reaction that does not include a potential modulator are optional, as the assays are highly uniform. Such optional control reactions are appropriate and increase the reliability of the assay. Accordingly, in some embodiments, the methods of the invention include such a control reaction. For each of the assay formats described, "no modulator" control reactions that do not include a modulator provide a background level of binding activity.

[0165] In some assays it will be desirable to have positive controls. At least two types of positive controls are appropriate. First, a known activator of a polypeptide or a polynucleotide of the invention can be incubated with one sample of the assay, and the resulting increase in signal resulting from an increased expression level or activity of a

SEQ ID NO:16 **human PPP3CA polypeptide sequence**

Protein sequence protein_id:gi306477

MSEPKAIDPKLSTTDRVVKAVPFPPSHRLTAKEVFDNDGKPRVDILKAHLMKEGRLEESVALRIITEG
ASILRQEKNLLDIDAPVTVCGDIHGQFFDLMKLFEVGGSPANTRYLFLGDYVDRGYFSIECVLYLWAL
KILYPKTLFLLRGNHECRHLTEYFTFKQECKIKYSERVYDACMDAFDCLPLAALMNQQFLCVHGGLSP
EINTLDDIRKLDRFKEPPAYGPMCDILWSDPLEDFGNEKTQEHFTHNTVRGCSYFYSYPAVCEFLQHN
NLLSILRAHEAQDAGYRMYRKSQTTGFPSLITIFSAPNYLDVYNNKAAVLKYENNVMNIRQFNCSPHP
YWLPNFMDVFTWSLPFVGEKVTEMLVNVLNICSDDELGSEEDGFDGATAAARKEVIRNKIRAIGKMAR
VFSVLREESESVLTLKGLTPTGMLPSGVLSGGKQTLQSATVEAIEADEAIKGFSPQHKITSFEEAKGL
DRINERMPPRRDAMPSDANLNSINKALTSETNGTDSNGSNSSNIQ

SEQ ID NO:17 **mouse PPP3CA nucleic acid sequence**

accession:J05479 coding sequence:76..1641

GCGCCGGTGCGGTCGGGGTGTGCAGTCGGACGGGACGAGCAGCGCGTCGCTGTCCCCCCCTCCCGGTG
GCTGGAGATGTCCGAGCCCAAGGCGATTGATCCCAAGTTGTCGACGACCGACAGGGTGGTGAAAGCCG
TTCCATTTCCACCAAGTCACCGGCTGACAGCAAAGGAAGTGTTTGATAATGATGGGAAACCTCGTGTG
GATATCTTAAAAGCACATCTCATGAAGGAGGGCAGGCTGGAAGAAAGTGTTGCATTGAGAATAATAAC
AGAGGGTGCTTCGATTCTCCGACAGGAAAAAAACTTGCTGGATATCGACGCACCAGTCACAGTTTGTG
GGGACATCCATGGACAATTCTTTGACTTGATGAAGCTCTTTGAAGTGGGAGGATCTCCTGCCAACACT
CGCTACCTCTTCTTAGGGGACTATGTTGACAGAGGGTACTTCAGTATCGAATGTGTGCTGTATTTGTG
GGCCTTGAAAATTCTTTACCCCAAAACACTGTTTTTACTTCGCGGAAACCATGAATGTAGGCACCTCA
CAGAGTATTTCACGTTTAAACAAGAATGTAAAATAAAGTATTCAGAACGCGTTTATGACGCCTGTATG
GATGCCTTCGACTGCCTTCCCCTGGCTGCGCTAATGAACCAGCAGTTCCTGTGTGTACACGGTGGTTT
GTCTCCAGAGATTAACACTCTAGATGACATCAGAAAATTAGACCGATTCAAAGAACCACCTGCTTATG
GGCCCATGTGTGACATCCTATGGTCAGACCCCCTGGAGGACTTTGGAAATGAGAAGACTCAGGAACAT
TTCACTCACAACACAGTCAGAGGCTGTTCGTACTTCTACAGTTACCCAGCTGTGTGTGACTTCCTGCA
GCACAATAATTTGTTGTCCATACTCCGCGCCCACGAAGCCCAGGATGCAGGGTACCGCATGTACAGGA
AAAGCCAAACAACAGGCTTCCCGTCTCTAATTACAATCTTCTCGGCACCAAATTACTTAGATGTGTAC
AATAACAAAGCTGCAGTGTTGAAGTACGAGAACAATGTGATGAACATCAGGCAGTTCAACTGCTCCCC
GCATCCGTACTGGCTCCCAAATTTCATGGATGTTTTCACCTGGTCGCTGCCATTTGTTGGGGAGAAAG
TGACTGAGATGCTGGTCAATGTTCTCAACATCTGCTCCGACGATGAACTGGGGTCAGAAGAAGATGGA
TTTGACGGAGCCACGGCCGCAGCCCGGAAGGAAGTCATCAGAAACAAGATCCGAGCAATAGGCAAAAT
GGCCAGAGTGTTCTCAGTTCTCAGAGAAGAGAGTGAGAGTGTCCTGACACTGAAGGGCCTGACCCCAA
CTGGCATGCTCCCCAGCGGAGTGCTCTCTGGCGGGAAACAGACTCTGCAAAGCGCTACTGTTGAGGCT
ATTGAGGCTGATGAAGCCATCAAAGGATTTTCACCACAACATAAGATCACTAGCTTCGAGGAGGCCAA
GGGCTTAGACCGAATTAACGAGAGGATGCCACCTCGCAGAGACGCCATGCCCTCTGACGCCAACCTTA